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Interaction of the Intron-Encoded Mobility Endonuclease I-*Ppo*I with Its Target Site

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Endonucleases encoded by mobile group I introns are highly specific DNases that induce a double-strand break near the site to which the intron moves. I-*Ppo*I from the acellular slime mold *Physarum polycephalum* mediates the mobility of intron 3 (Pp LSU 3) in the extrachromosomal nuclear ribosomal DNA of this organism. We showed previously that cleavage by I-*Ppo*I creates a four-base staggered cut near the point of intron insertion. We have now characterized several further properties of the endonuclease. As determined by deletion analysis, the minimal target site recognized by I-*Ppo*I was a sequence of 13 to 15 bp spanning the cleavage site. The purified protein behaved as a globular dimer in sedimentation and gel filtration. In gel mobility shift assays in the presence of EDTA, I-*Ppo*I formed a stable and specific complex with DNA, dissociating with a half-life of 45 min. By footprinting and interference assays with methidiumpropyl-EDTA-iron(II), I-*Ppo*I contacted a 22- to 24-bp stretch of DNA. The endonuclease protected most of the purines found in both the major and minor grooves of the DNA helix from modification by dimethyl sulfate (DMS). However, the reactivity to DMS was enhanced at some purines, suggesting that binding leads to a conformational change in the DNA. The pattern of DMS protection differed fundamentally in the two partially symmetrical halves of the recognition sequence.

More than 100 group I introns, which are defined by a common RNA secondary structure and mechanism of splicing, have been identified in the organellar genomes of lower eucaryotes (for reviews, see references 4 and 29). Several of these introns also have been found in bacteria, bacteriophage, and extrachromosomal nuclear ribosomal DNA (rDNA). The RNAs of many group I introns are capable of self-splicing in vitro. Some group I introns behave as mobile elements, as first described for the optional omega intron (Sc LSU 1) of *Saccharomyces cerevisiae* (9). Additional mobile group I introns have been found in yeast mitochondria (32, 45, 53), bacteriophage (42), *Chlamydomonas* chloroplasts (11, 23), *Chlamydomonas* mitochondria (8, 26), and nuclear rDNA of slime molds (16, 36). In each of these cases, the mobile intron is copied into an intron$^-$ allele of the gene in which it resides, converting it to intron$^+$ by gene conversion initiated at a double-strand break near the site of insertion. This process, known as intron homing (10), is mediated by an endonuclease encoded within the intron, which recognizes and cleaves the intron$^-$ DNA within a few nucleotides of the insertion site (for reviews, see references 22 and 33). In three cases, the procaryotic enzymes I-*Tev*I, I-*Tev*II, and I-*Tev*III, the DNA cut is somewhat more removed, 13 to 26 bp from the point of intron insertion (for a review, see reference 33). In any case, the homology between the intron$^-$ allele and the DNA sequences flanking the intron in the intron$^+$ allele allows the break to be repaired via a gene conversion event which uses the intron$^+$ allele as a template. Since intron insertion renders the DNA resistant to cleavage, gene conversion is unidirectional, and the intron homing process is eventually driven to completion.

The fact that intron homing is site specific is accounted for in part by the specificity of the endonuclease and in part by the requirement for homologous flanking sequences in the gene conversion process. The specificity of a few intron-encoded endonucleases has been investigated experimentally. The yeast mitochondrial enzymes I-*Sce*I and I-*Sce*II recognize 18-bp sequences (7, 54), and the algal chloroplast protein I-*Cre*I is reported to require a 24-bp sequence for cleavage (51). The phage enzyme I-*Tev*I recognizes ca. 40 bp of DNA (3). Large recognition sequences are also required by the yeast endonucleases, encoded in the nucleus, Endo.*Sce*I (26 bp) (17) and HO endonuclease (ca. 24 bp) (38). These two enzymes, I-*Sce*I, I-*Sce*II, I-*Sce*III, I-*Sce*IV, I-*Cre*I, I-*Ceu*I, I-*Csm*I, and I-*Dmo*I all are also related by the presence of a conserved peptide, LAGLI-DADG (for a review, see reference 22). In most cases, this motif is present twice, separated by about 100 amino acids. The large recognition sequences of these enzymes lack the strict dyad symmetry characteristic of type II restriction enzymes targets. In the few cases in which mutagenesis of the DNA recognition sequence has been carried out, intron-encoded endonucleases and HO nuclease were found to tolerate, to different degrees, individual point mutations within their recognition sequences (2, 3, 7, 39, 44, 54). Thus, the specificity requirements appear strikingly different from those of restriction enzymes. However, compared with restriction enzymes, little is known about the protein-DNA interactions of these proteins.

I-*Ppo*I mediates homing of intron 3 (Pp LSU 3) in the extrachromosomal nuclear rDNA of the acellular slime mold *Physarum polycephalum* (36). Pp LSU3 is one of only two known mobile introns that exist in the nucleus. The other, Di SSU 1, is also found in the extrachromosomal rDNA of an acellular slime mold (16). I-*Ppo*I has been expressed in several systems, including rabbit reticulocyte lysates, *Escherichia coli* (34), and *S. cerevisiae* (37). In vitro and in *E. coli*, either of two methionine codons may serve to initiate translation. Functional enzyme has been synthesized both from a coding sequence found entirely within the intron, beginning at an AUG 14 nucleotides from the 5′ splice site

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and yielding an 18-kDa protein, and from an in-frame AUG located 53 nucleotides into the upstream exon and yielding a 20-kDa version of I-*Ppo*I (12, 34). Which form is synthesized in *P. polycephalum* is unknown. Thus far, I-*Ppo*I is the only characterized intron-encoded endonuclease that is expressed in the nucleus. I-*Ppo*I also differs from most other intron-encoded endonucleases in that it both lacks the LAGLI-DADG peptide and it cleaves in a sequence with considerable symmetry. Ten of fifteen base pairs of the homing site contribute to an imperfect palindrome.

We showed previously that I-*Ppo*I creates a four-base staggered, 5′-recessed double-strand break near the point of intron insertion in the intron$^-$ DNA (34). We have now identified the minimal target sequence for the endonuclease by deletion analysis, measured the dissociation rate of the protein from the DNA, and determined that the protein is a dimer. We have also characterized the protein-DNA interaction by footprinting, interference, and methylation protection studies.

MATERIALS AND METHODS

Deletion analysis and size determination. To define the recognition sequence for I-*Ppo*I, a nested series of deletions surrounding the cleavage site was constructed from the target plasmid p42. This plasmid contains 38 bp of DNA corresponding to the intron homing site, inserted into the *Eco*RI and *Pst*I sites of pBluescript KS (34). The deletion mutants were created with a nested deletion kit (Pharmacia) as instructed by the manufacturer, and all mutants were sequenced. In some cases, a 1,000-fold molar excess of *Eco*RI linkers (New England Biolabs) was added before religation of the plasmid. To assay the ability of the mutants to be cleaved, approximately 1 μg of each plasmid was linearized with *Ava*II and then incubated at 37°C with 9 or 90 ng of purified 20 kDa I-*Ppo*I (Promega Corp.) for 1 or 2 h, respectively, in buffer A (50 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM $MgCl_2$). The amount of DNA cleavage was measured by densitometric analysis of the photographic negative, using a Bio-Rad model 620 video densitometer.

To determine the native molecular weight of I-*Ppo*I, 10 μg of the endonuclease was sedimented in a 4-ml 10 to 30% glycerol gradient in buffer B (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride [PMSF]). Included in the gradient were 25 μg each of bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and cytochrome *c*. Centrifugation was carried out in a Beckman SW60 rotor at 50,000 rpm for 40 h at 4°C. Of the 200-μl samples taken from the top of the gradient, 40 μl was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and silver staining (43). Gradient fractions containing I-*Ppo*I activity were detected by assaying for cleavage of linearized p42. I-*Ppo*I activity in 2-μl samples was measured after 25-fold dilution in buffer C (50 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 0.2 mM PMSF, 10% glycerol, 1 mg of bovine serum albumin per ml) and 30 min of incubation at 37°C with 1 μg of linearized p42. The DNA was electrophoresed on a 1.5% agarose gel along with 2 μg of the 1-kb DNA ladder.

Gel filtration chromatography was performed at a flow rate of 3.9 ml/min, using a Bio-Gel P-100 column (37 by 1 cm; Bio-Rad) and 13.5 ng of I-*Ppo*I in buffer D (50 mM Tris-HCl [pH 7.0], 150 mM NaCl, 1 mM dithiothreitol, 0.2 mM PMSF). Size standards included 1 mg each of blue dextran, bovine serum albumin (3.5 nm), ovalbumin (2.8 nm), cytochrome *c* (1.7 nm), carbonic anhydrase (2.0 nm), and aprotinin (1.0 nm). Protein peaks were detected by SDS-polyacrylamide gel electrophoresis of column fractions and staining with Coomassie brilliant blue or immunoblotting with anti-I-*Ppo*I serum. Each 480-μl fraction was diluted 1:100 in buffer C, and 2 μl was used in I-*Ppo*I activity assays for 1 h as described for deletion analysis.

Dissociation constant, footprinting, and interference assays. The 86-bp *Xho*I-*Xba*I fragment of p42, which contains the I-*Ppo*I homing site, was used for gel mobility shift assays in all of the DNA binding experiments described below. The fragment was labeled at either end with α-^{32}P-labeled deoxyribonucleotide triphosphates and the Klenow fragment of DNA polymerase. Gel mobility shift assays were performed essentially as described previously (1). For the gel mobility shift assay shown, I-*Ppo*I was incubated in 20 μl with approximately 1 ng of DNA at 37°C for 15 min in buffer E (10 mM Tris-HCl [pH 7.5], 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 300 μg of bovine serum albumin per ml). The total amount of enzyme used was 6.5 pg to 6.5 ng. In some reactions, poly(dI-dC) (Boehringer Mannheim Biochemicals) was included at a concentration of 0.1 mg/ml. Samples were immediately electrophoresed on a 4% polyacrylamide gel containing 7 mM Tris-HCl (pH 8.0), 3.3 mM sodium acetate, 1 mM EDTA, and 2.5% glycerol, and the gel was subjected to autoradiography.

To determine the rate of dissociation, 40 pg of I-*Ppo*I was used to bind 30 pg of end-labeled DNA in the absence of poly(dI-dC). The binding reactions were performed essentially as described above in a final volume of 20 μl. After 15 min, approximately 5 ng of cold homologous DNA was added, and incubation was continued for various lengths of time. The samples were immediately electrophoresed on a 4% polyacrylamide gel, and the bands on the dried gel were detected and quantified with a BetaScope 603 blot analyzer. The exponential curve shown was fit to the data points by using Genplot data analysis software.

Footprinting and interference studies with methidiumpropyl-EDTA (MPE)-iron (II) [MPE · Fe(II)] were carried out essentially as described previously (41). For MPE · Fe(II) footprinting, 26 ng of I-*Ppo*I was used to bind 4 ng of DNA under conditions described for gel mobility shift assays [including poly(dI-dC)]. MPE, $Fe(NH_4)_2(SO_4)_2$ solution, and dithiothreitol were added to final concentrations of 2.85, 5, and 50 mM, respectively, and incubation was continued at 37°C for 10 min. The protein-DNA complex was eluted from a preparative band shift gel in 0.5 M ammonium acetate (pH 8.0)–1 mM EDTA. To each sample, 3 μg of sonicated salmon sperm DNA were added as carrier, and the DNA was extracted with phenol and chloroform and precipitated with ethanol. The DNA ladder was created under similar conditions but in the absence of I-*Ppo*I and without gel purification of the DNA. The A+G sequencing ladder of the *Xba*I-*Xho*I fragment was generated by chemical cleavage of the DNA (27). All DNA samples were resuspended in formamide, heat denatured, and electrophoresed on a 7 M urea–10% polyacrylamide gel and subjected to autoradiography. The autoradiograms were analyzed by densitometry. For MPE · Fe(II) interference, the DNA was first treated with MPE · Fe(II) in the absence of I-*Ppo*I (under conditions as described for footprinting) and then precipitated with ethanol. I-*Ppo*I was bound to the punctured DNA, and the bound and free DNAs were purified as described for MPE · Fe(II) footprinting.

Methylation protection experiments with dimethyl sulfate (DMS) were performed essentially as described previously (46). Approximately 12 ng of DNA was bound under the

conditions described above [including poly(dI-dC)] in a volume of 60 μl. The reaction mixtures contained either 26 ng of I-*Ppo*I or no I-*Ppo*I. The DNA was methylated with 0.5% (vol/vol) DMS for 5 min at room temperature, and the reaction was stopped by the addition of 2-mercaptoethanol to 1%. The reaction volumes were brought to 200 μl with 100 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 μg of yeast tRNA, and the samples were treated with proteinase K. The resulting DNA was purified by organic extractions and ethanol precipitation, and the DNA backbone was broken at methylated bases with alkali. After precipitation with ethanol and resuspension in formamide, the DNA was electrophoresed on a 7 M urea–10% polyacrylamide sequencing gel along with its A+G sequencing ladder and subjected to autoradiography. The resulting autoradiogram was analyzed by densitometry as described above, and the data were manipulated by using 1-D Analyst software. Bands whose estimated intensity differed from that of naked DNA by >50% represent bases classified as strongly protected or hypersensitive. Bases considered partially protected or showing an increase in sensitivity are those whose bands were estimated to differ in intensity by 10 to 50% from naked DNA.

RESULTS

A 13- to 15-bp sequence is required for cleavage. To determine the recognition sequence for I-*Ppo*I, we constructed a series of deletion mutants surrounding the cleavage site. These mutants were derived from the target plasmid p42, which contains 38 bp of DNA corresponding to the I-*Ppo*I homing site. Each of the mutant plasmids was assayed for its ability to be cleaved by I-*Ppo*I (Fig. 1). Cleavage assays were carried out first under conditions in which approximately 70% of the DNA was cleaved (9 ng of I-*Ppo*I) (Fig. 1B). In separate assays, the I-*Ppo*I concentration was increased 10-fold (90 ng of I-*Ppo*I) and the time of incubation was increased twofold in order to detect low-level cleavage. All plasmids containing a 13- to 15-bp sequence covering the cleavage site were readily cleaved. This sequence is partially symmetrical about the cleavage site and is part of a larger sequence that is identical in all eucaryotes (Fig. 1C). Plasmids p42-5 and p42+6 were not cleaved under conditions in which we estimate a 500-fold-reduced rate would have led to detectable cleavage. However, plasmids p42-4, p42+3, and p42+5 were cleaved at low rates (50-, 300-, and 300-fold lower than wild type, respectively), even though the deletions in these three plasmids extend farther toward the cleavage site than those in p42−5 and p42+6. (The cleavage products of plasmids p42+3 and p42+5 are barely detectable.) We hypothesize that the three slowly cleaving sequences are recognized by I-*Ppo*I because they contain key positions of identity with the wild-type sequence, which do not occur in p42−5 and p42+6. Specifically, the C residues at −7 and +8 may be important for recognition by I-*Ppo*I. Although deletion analysis implicates a 13- to 15-bp sequence, it cannot be excluded that sequences flanking these 13 to 15 bp also contribute to recognition by the enzyme.

I-*Ppo*I is a dimer in solution. While most restriction enzymes are known to function as dimers, the native size has been reported for only two intron-encoded endonucleases. The yeast mitochondrial enzyme I-*Sce*I was reported to behave as a monomer in solution (31), while by gel filtration, I-*Sce*II was found to be dimeric (55). The comparatively extensive dyad symmetry within the I-*Ppo*I recognition sequence suggests that this enzyme may resemble restriction enzymes and function as a homodimer. Sedimentation and gel filtration were used to determine the native molecular weight of I-*Ppo*I. For sedimentation analysis, the purified enzyme was centrifuged in a 10 to 30% glycerol gradient along with a set of molecular weight standards. I-*Ppo*I was detected in the resulting fractions by silver staining of SDS-polyacrylamide gels (Fig. 2A) as well as by enzymatic assay (Fig. 2B). The endonuclease migrated with a sedimentation coefficient of ca. 3.2, with the peak of protein and the peak of enzymatic activity coinciding. The sedimentation rate is that expected for a 34- to 37-kDa globular protein. Very little cleavage was detectable where an I-*Ppo*I monomer would be expected to sediment (fraction 9). These results suggest that I-*Ppo*I is dimeric.

Since an elongated shape would lead to an underestimate of native molecular weight in sedimentation, we also determined the size of I-*Ppo*I by gel filtration chromatography. The enzyme eluted with a derived Stokes radius of 2.5 to 2.7 nm, which corresponds to a molecular size of 34 to 39 kDa for a globular protein (47) (Fig. 3A). Only low levels of endonuclease activity were detectable where an I-*Ppo*I monomer would elute (fraction 37), which we interpret to be due to slight trailing of the protein throughout the column. Taken together, the sedimentation and chromatography data imply that I-*Ppo*I is approximately a globular dimer at the protein concentrations used in these experiments.

Gel mobility shift and dissociation rate. As a preliminary to the DNA binding studies described below, I-*Ppo*I was assayed for its ability to specifically bind its target DNA. These experiments were carried out with an 86-bp end-labeled DNA fragment in the presence of EDTA to inhibit cleavage of the DNA during the assay. The endonuclease readily formed a stable and specific protein-DNA complex under these conditions (Fig. 4A). When added in molar excess over the DNA, I-*Ppo*I formed more slowly migrating complexes with the DNA. These higher-order complexes could be suppressed by addition of poly(dI-dC) and thus are likely to represent nonspecific interaction with DNA. Poly(dI-dC) also appeared to decrease formation of the specific complex, suggesting that I-*Ppo*I has some nonspecific affinity for DNA. The appearance of a second shifted complex at the 1:1 protein-to-DNA ratio (lane 3) probably reflects an overestimation of the DNA concentration. The nature of the two species of uncomplexed DNA is not known (lane 1).

To further characterize the specific protein-DNA complex, we measured the rate of dissociation of I-*Ppo*I from its target DNA. After binding of the endonuclease to radioactive DNA, as for the gel mobility shift assay, the nonradioactive homologous fragment was added in large excess. The amount of labeled complex then was determined as a function of time (Fig. 4B). The dissociation appeared to be first order, with a half-life of the complex under these conditions of 45 min, corresponding to a rate of 2.58×10^{-4} s^{-1}. This rate of dissociation is comparable to that for *Eco*RI (5.3×10^{-4} s^{-1}) and for the *Eco*RI mutant Gly-111 (1.3×10^{-4} s^{-1}) from pBR322 DNA (19).

MPE · Fe(II) footprinting and interference analysis and DMS protection studies. To learn how I-*Ppo*I contacts its target DNA, we performed several types of footprinting and interference studies. For footprinting with MPE · Fe(II), I-*Ppo*I was bound to the singly end-labeled 86-bp *Xba*I-*Xho*I fragment of p42. The DNA then was treated with the intercalating agent MPE · Fe(II), which breaks the phosphodiester backbone and removes a nucleoside where the DNA is not protected by the bound protein (14). The

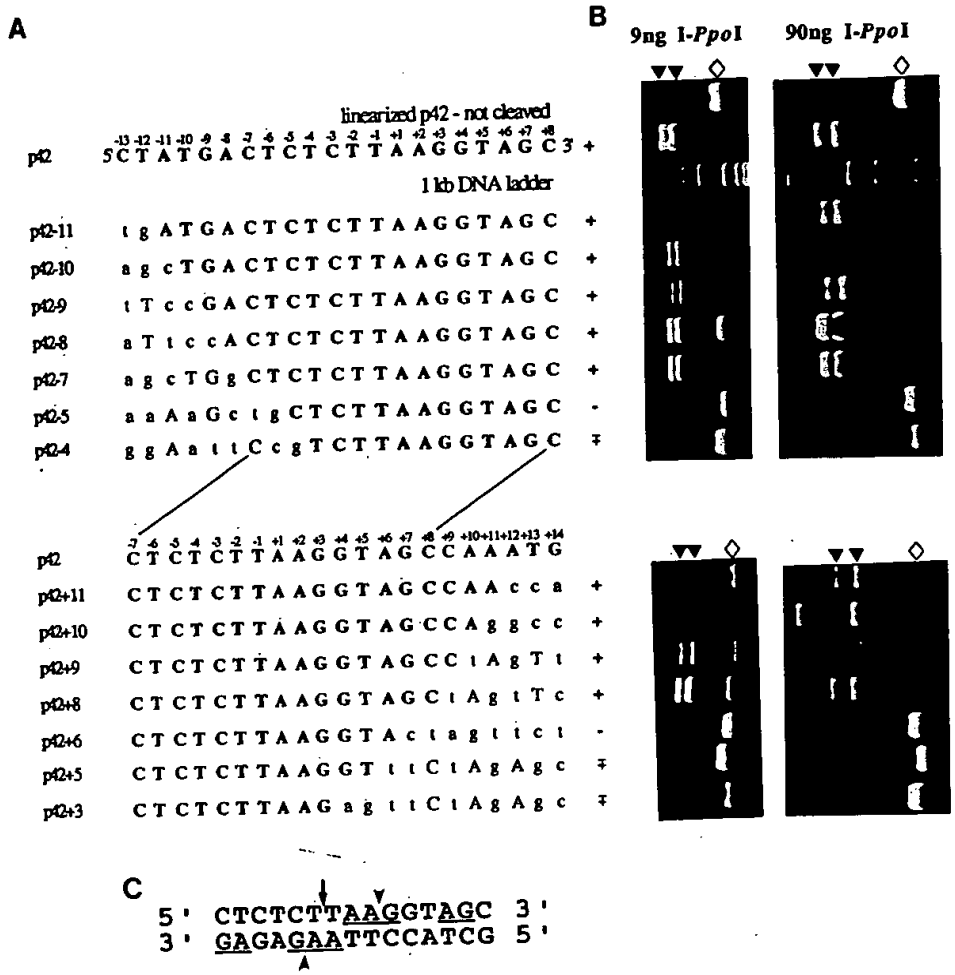


FIG. 1. Definition of the recognition sequence. (A) The name of each deletion mutant, constructed from plasmid p42, is given on the left. Numbering is from the center of the cleavage site and corresponds to the number of wild-type nucleotides (capital letters) remaining after deletion. Lowercase letters represent mutant nucleotides. Variation in the sizes of the plasmids results from differences in the amount of DNA deleted and in the number of *Eco*RI linkers inserted. To the right of each sequence is a summary of the cleavability of that plasmid: cleaved at approximately the same rate as p42 (+), no detectable cleavage under the conditions used (−), or cleaved at a substantially slower rate than wild type (±). (B) Cleavage assays for each deletion mutant, using either 9 or 90 ng of I-*Ppo*I. The diamond designates the linearized plasmid; triangles indicate the cleavage products of ca. 1.3 and 1.5 kb. Also included are the linearized plasmid p42 and the 1-kb DNA ladder (Gibco BRL) as size markers. (C) Summary of the recognition sequence. The cleavage site (arrowheads) and point of intron insertion (arrow) are shown in the 13- to 15-bp recognition sequence. Nucleotides which contribute to the symmetry of the recognition sequence are underscored.

migration of protected DNA was compared with a DNA ladder generated in the absence of the endonuclease and with a sequencing ladder derived by chemical cleavage of the same fragment (Fig. 5A). I-*Ppo*I was found to protect a 22- to 24-bp sequence on each strand of the DNA, centered nearly symmetrically about the cleavage site. Thus, it appears that I-*Ppo*I makes contacts with the DNA about 4 bp beyond the sequence identified by deletion analysis. The intense band in the middle of each footprint, which corresponds exactly to the sites of enzymatic cleavage in the presence of Mg^{2+}, was evident even when the experiment was performed in the absence of MPE · Fe(II) (data not shown). Thus, this band appears to have resulted from a low level of residual endonuclease activity even in the presence of EDTA. (Because chemical sequencing removes a nucleotide during the reaction, the I-*Ppo*I cleavage products comigrated with a band in the sequencing ladder [−2 or +2] that is one nucleotide from the cleavage site.)

A modification of MPE · Fe(II) footprinting was used to further characterize the interaction of I-*Ppo*I with its DNA substrate. In MPE · Fe(II) interference experiments, the DNA is first treated with MPE · Fe(II) to puncture the DNA. Protein subsequently is bound to the DNA, and the complexed and free DNA molecules are separated by electrophoresis, purified, and analyzed. The intensity of bands which represent MPE · Fe(II)-mediated cleavage at nucleotides that are required for binding is diminished in the bound fraction and enhanced in the free fraction. When I-*Ppo*I binding was analyzed in this way, the same sequence as that found by footprinting was identified (Fig. 5B). Thus, even the nonspecific protein-DNA contacts at the edges of the footprint are required for binding.

To gain more detailed information about protein-DNA contacts, I-*Ppo*I-DNA complexes were probed by DMS protection. DMS modifies DNA more subtly than MPE · Fe(II) does. It methylates guanine at the N-7 posi-

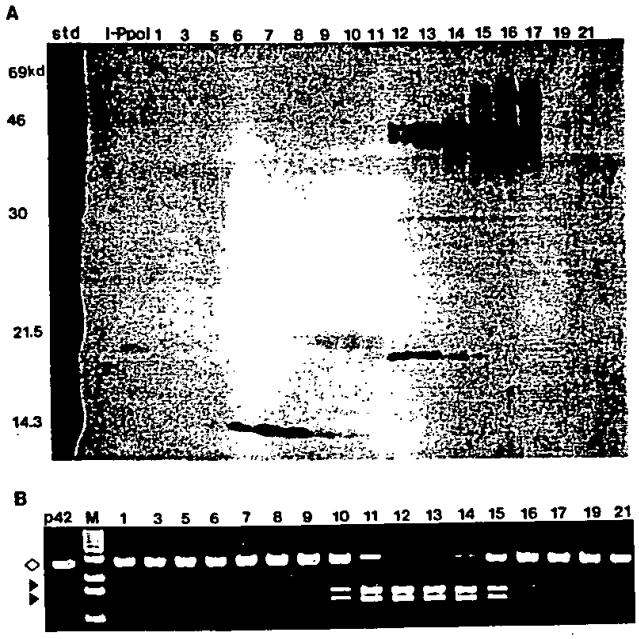


FIG. 2. Sedimentation analysis. I-*Ppo*I was sedimented through a glycerol gradient along with a set of molecular size standards: bovine serum albumin (66 kDa, 4.5S), ovalbumin (46 kDa, 3.4S), carbonic anhydrase (29 kDa, 3.06S), trypsin inhibitor (21.5 kDa, 2.3S), and cytochrome *c* (12.4 kDa, 1.83S). (A) Protein peaks were detected by SDS-polyacrylamide gel electrophoresis (gradient fraction numbers are indicated). As size markers, the gel also included 450 ng of purified I-*Ppo*I and prestained molecular weight markers (std; Amersham). Sizes of the prestained markers are indicated at the left. The arrow indicates the location of I-*Ppo*I. (B) Gradient fractions were used to assay for cleavage of linearized p42. As molecular weight standards, the 1-kb DNA ladder was included. The diamond designates the linearized plasmid, and triangles indicate the cleavage products.

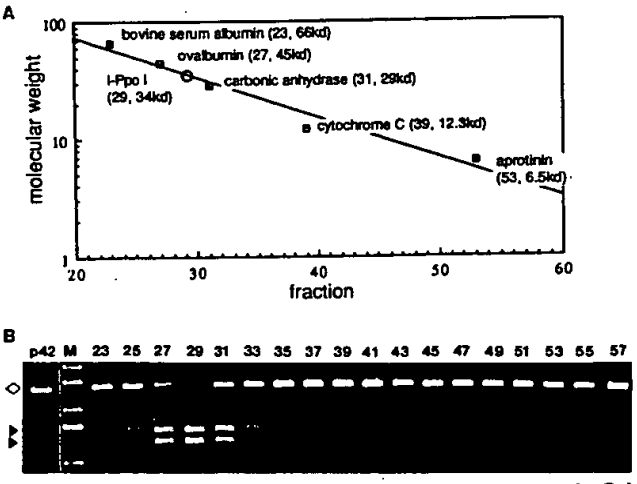


FIG. 3. Gel filtration. Gel filtration was performed with a Bio-Gel P-100 column along with a set of size standards. (A) The fraction in which each protein was eluted (shown in parentheses, followed by the molecular size) was determined by SDS-polyacrylamide gel electrophoresis. (B) Activity assays of gel filtration column fractions. Included are the linearized plasmid p42 and the 1-kb DNA ladder (M). The diamond indicates the linearized plasmid, and triangles designate the cleavage products.

tion, which lies in the major groove, and adenine at the N-3 position, which lies in the minor groove. Protein bound to DNA can protect against methylation. Thus, the pattern of protection can lead to inferences about the interaction of the protein with the two grooves of the DNA helix. In these experiments, I-*Ppo*I was bound to a uniquely end-labeled fragment under conditions in which the protein was in molar excess, and the complexes thus formed were incubated with DMS. Then the DNA backbone was broken at the methylated purines by treatment with alkali. Electrophoresis of the denatured DNA and densitometric analysis revealed the pattern of protection.

Protection of guanines was detected on the top strand at positions −9, +3, +4, and +7 (Fig. 6A) and on the bottom strand at guanines −7, −5, +8, and +9 (Fig. 6B). These bases encompass all but one guanine in the region identified by MPE · Fe(II) footprinting and interference studies. Protection of the guanines from positions +3 to +9 indicates that I-*Ppo*I occupies the major groove for more than a half-turn of the helix, suggesting that it approaches the DNA from both faces (for a summary, see Fig. 6C). Strikingly, the guanine at position −3 on the bottom strand was found not to be protected. Densitometric analysis of the band corresponding to this base showed that in the presence of I-*Ppo*I, this guanine became slightly more sensitive to methylation than in naked DNA. Thus, it appears that I-*Ppo*I does not occupy the major groove immediately at the cleavage site on the bottom strand.

Protection of adenines was less strong than protection of guanines, suggesting that the most significant contacts are made in the major groove. Nonetheless, protection of adenines was observed over the length of the binding site defined by MPE · Fe(II) footprinting and interference. On the top strand, adenines −8, +1, +6, +10, +11, and +12 were partially protected by binding of I-*Ppo*I (Fig. 6A). As for guanines, protection of adenines was not limited to simple blocks of 5 bp, suggesting that the endonuclease contacts the DNA from both sides of the double helix. A prominent feature of the adenine protection pattern is the hypersensitive adenine at position +5 on the bottom strand. This increase in methylation is indicative of a significant perturbation of the DNA in that region. The adenine at position −4 on the bottom strand also showed a slight increase in methylation in the presence of I-*Ppo*I. This observation, combined with the lack of protection of adenines −2 and −1, implies that the endonuclease does not occupy the minor groove immediately around the cleavage site on the bottom strand. As with the MPE · Fe(II) studies, low-level enzymatic cleavage of the DNA accounts for the increased intensity at adenine −2 on the bottom strand. Although it does not appear on this scan, a similar increase in intensity was seen in other experiments at adenine +2 on the top strand.

DISCUSSION

We have characterized the binding of I-*Ppo*I to its DNA homing site. By deletion analysis, the DNA target is a 13- to 15-bp partially symmetric sequence centered on the cleavage site. This sequence is identical in the nuclear rDNA of all eucaryotes. Sedimentation and gel filtration data imply that I-*Ppo*I is a globular homodimer. In the presence of EDTA, the enzyme forms a specific complex with target DNA. From MPE · Fe(II) footprinting and interference as well as DMS protection studies, I-*Ppo*I can be inferred to interact with

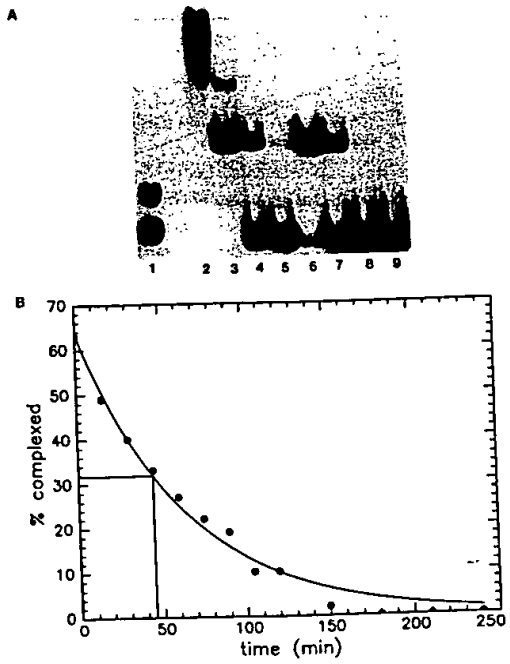


FIG. 4. Gel mobility shift assay and dissociation rate. (A) I-*Ppo*I was bound to the *Xba*I-*Xho*I fragment of p42 in the absence (lanes 2 to 5) or presence (lanes 6 to 9) of poly(dI-dC). The reaction mixtures contained various amounts of the endonuclease to give a range of molar ratios of I-*Ppo*I to DNA (indicated below in parentheses). The amount of I-*Ppo*I per reaction was 0 ng (lane 1), 6.5 ng (10:1; lanes 2 and 6), 650 pg (1:1; lanes 3 and 7), 65 pg (0.1:1; lanes 4 and 8), or 6.5 pg (0.01:1; lanes 5 and 9). (B) I-*Ppo*I was bound to end-labeled DNA as described in Materials and Methods. The percentage of protein-DNA complex remaining after the addition of nonradioactive competitor DNA is shown as a function of time. The vertical line shows the $t_{1/2}$.

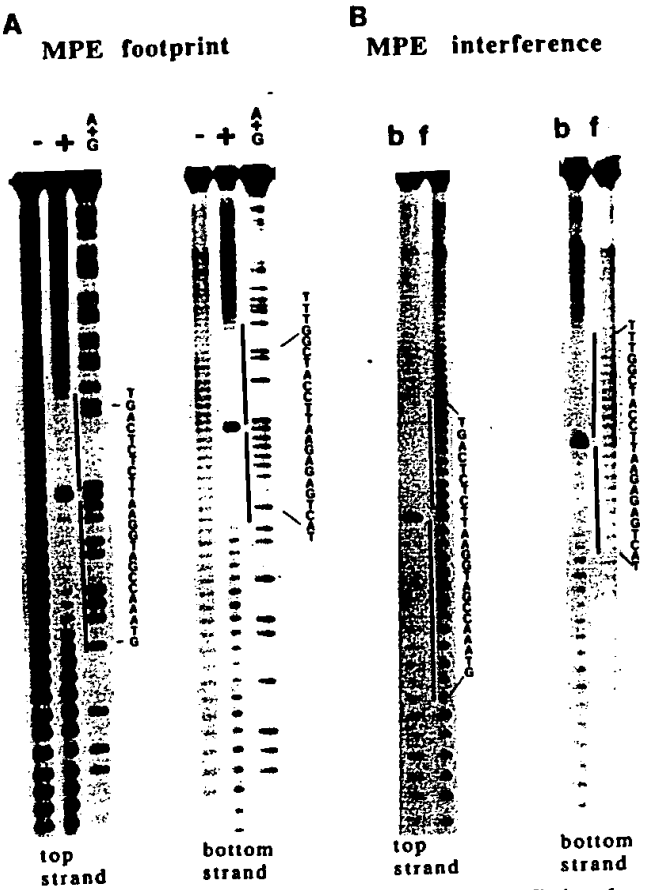


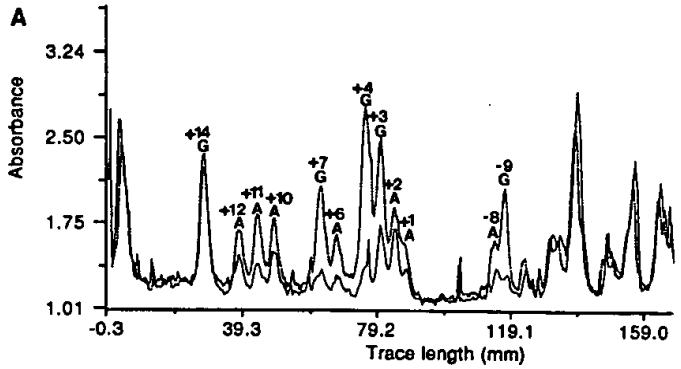
FIG. 5. MPE · Fe(II) footprinting and MPE · Fe(II) interference. (A) The *Xba*I-*Xho*I fragment of p42 was treated with MPE · Fe(II) to create a DNA ladder (−) or first bound by I-*Ppo*I and then treated with MPE · Fe(II) (+). The same DNA fragment was subjected to chemical sequencing (A+G). The boundaries of the footprint are demarcated by bars. (B) The DNA was first treated with MPE · Fe(II) and then used in the binding reactions. The bound (b) and free (f) DNAs were purified from preparative band shift gels prior to electrophoresis under denaturing conditions.

approximately 23 bp of the DNA via both the major and minor grooves of the helix. Binding to DNA is not entirely symmetric and appears to induce conformational changes in the DNA.

To date, the recognition sites have been reported for only a few of the several known intron-encoded endonucleases. The yeast mitochondrial enzymes I-*Sce*I and I-*Sce*II each recognize 18-bp sequences (7, 44, 54), while the algal chloroplast endonuclease I-*Cre*I requires a 24-bp sequence for cleavage (51). I-*Tev*I has a relatively large recognition sequence of ca. 40 bp (3). The recognition sequence defined by deletion analysis of the I-*Ppo*I target site is slightly smaller than those which have been defined thus far and differs in that it contains considerable elements of twofold symmetry. Equally extensive symmetry in the homing site is found only in the recognition sequence for I-*Cre*I.

For the intron-encoded endonucleases that have been examined to date, mutagenesis of the recognition sequence revealed that those enzymes are capable of cleaving noncognate sequences. Extensive mutagenesis of the recognition sequence for I-*Sce*II indicated that numerous point mutations are permitted, although most of them cause some reduction in the efficiency of cleavage (44, 54). While the sequence requirements of I-*Sce*I appear to be significantly more stringent, several substitutions within that enzyme's recognition sequence failed to abolish cleavage (7). HO nuclease also tolerates multiple point mutations in its 24-bp recognition sequence (38, 39). The most permissive enzyme appears to be I-*Tev*I, which tolerates any single substitution within a 48-bp region surrounding the insertion point (2, 3).

Like other intron-encoded mobility endonucleases, I-*Ppo*I appears to tolerate sequence changes within its binding site. Preliminary results suggest that at high pH, most oligonucleotides containing single substitutions within the recognition sequence are cleaved to the same extent as oligonucleotides containing the wild-type sequence. In addition, while several oligonucleotides containing two substituted bases resulted in severely reduced cutting by I-*Ppo*I, other doubly substituted oligonucleotides were cleaved at levels similar to the wild-type sequence (21). Despite the tolerance for noncognate sequences, I-*Ppo*I maintains a high degree of specificity. Incubation of yeast chromosomes with I-*Ppo*I in vitro caused cleavage only of the rDNA repeats on chromosome XII (24, 35), indicating that in wild-type yeast strains, the only sequence recognized is in rDNA. Expression of the endonuclease in *S. cerevisiae* is lethal, presumably as a result of double-strand breaks in the rDNA in vivo. Mutations in yeast rDNA at position −3 (C→A) or at positions +2



C

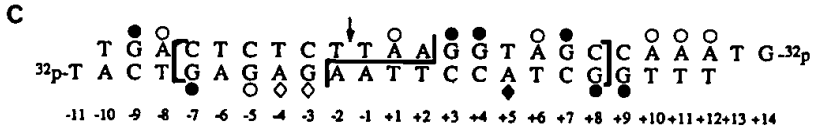


FIG. 6. DMS protection. The *Xba*I-*Xho*I fragment of p42 was incubated with I-*Ppo*I under standard binding conditions and subsequently methylated and depurinated as described in Materials and Methods. The denatured samples were electrophoresed on a 10% sequencing gel, which was subjected to autoradiography and densitometry. (A) Densitometric scans for the top strand. The superimposed lines represent DNA incubated without I-*Ppo*I (dashed line) or DNA incubated with 26 ng of I-*Ppo*I (solid line). (B) Densitometric scans for the bottom strand. (C) Summary of DMS protection results. The sequence shown is protected in MPE footprinting studies, and the brackets demarcate the 13- to 15-bp sequence defined by deletion analysis. The staggered line indicates the cleavage site, and the arrow shows the point of intron insertion. The bases which are differentially sensitive to DMS are indicated by filled circles (strong protection), open circles (partial protection), filled diamonds (hypersensitivity), or open diamonds (increased sensitivity).

and +3 (AG→GT) abolish cleavage by I-*Ppo*I in vitro and presumably in vivo (37). Saturation mutagenesis of the entire DNA binding site could provide more information about the actual specificity of the endonuclease.

Our studies on the protein-DNA interactions relied on the inhibition of I-*Ppo*I activity by EDTA. While many proteins retain their DNA binding specificity in the absence of divalent metal ions, e.g., the restriction endonuclease *Eco*RI (15), others do not. For example, *Eco*RV requires metal ions to preferentially bind its recognition sequence (48, 50). Most intron-encoded endonucleases have not been examined to determine whether they require divalent cations to specifically bind their target DNAs. In gel mobility shift assays, I-*Tev*I binds its substrate DNA in the presence of EDTA, but two complexes are detected (2). I-*Ppo*I forms a stable and specific complex with its homing site in the presence of the chelator. The dissociation rate of this complex, 2.58×10^{-4} s^{-1} (corresponding to a half-life of 45 min), is comparable to the dissociation rate for *Eco*RI from pBR322, 5.3×10^{-4} s^{-1} (19). Other kinetic data also suggest that I-*Ppo*I is similar to restriction enzymes. The turnover rate of I-*Ppo*I (2.6 min^{-1} [24]) is more similar to that for *Eco*RI (3.4 min^{-1} [30]) than it is to those of other intron-encoded endonucleases (0.058 min^{-1} for I-*Sce*I [31] and 0.011 min^{-1} for I-*Sce*II [55]).

While the specific recognition sequences have been defined for a few of the intron-encoded endonucleases, footprinting studies have been reported for only the procaryotic enzyme I-*Tev*I (2, 3). We have examined the binding of I-*Ppo*I to its homing site by using the intercalating and puncturing agent MPE · Fe(II). Although deletion analysis implicated a 13- to 15-bp sequence, the enzyme also interacts with 3 to 5 bp flanking this sequence, as evidenced by protection against MPE · Fe(II). This mode of interaction might be compared with that of *Eco*RI, which specifically recognizes 6 bp yet is closely associated with at least 10 bp (25). We interpret the observation that MPE · Fe(II) cleavage in these flanking sequences also abrogated binding of I-*Ppo*I to mean that those nucleotides play a fundamental

role in anchoring the protein on the DNA. It is not clear whether these contacts are at the sugar-phosphate backbone or at the individual bases.

Of the nucleotides identified in MPE · Fe(II) footprinting, most also were protected from modification by DMS. The protected purines include G−7 and G+8, implicated by deletion analysis as being important for recognition. As is the case for Endo.*Sce*I (17), both the major and minor grooves are implicated as sites of contact between the protein and DNA. The observation that protection of guanines was stronger and more extensive than that of adenines, however, implies that the predominant protein-DNA interactions take place in the major groove of the helix. Neither protection of guanines nor protection of adenines is limited to simple blocks of five nucleotides, indicating that I-*Ppo*I approaches both grooves from both faces of the helix. Unlike the protection patterns for some repressors (5, 6, 52), protection of the minor groove by I-*Ppo*I is not limited to the space intervening successive occupied major grooves along one face of the helix. Conceivably, protection of the minor groove may result from contact by flexible arms of the endonuclease which wrap around the DNA. This type of interaction would resemble that of homeodomain proteins such as engrailed (20) and antennapedia (40). Those proteins bind predominantly in the major groove yet reach around the DNA to contact the minor groove.

In our studies, significantly different patterns of protection were observed on the two strands of the DNA near the cleavage site. On both strands, the cleavage site is in the middle of a stretch of four purines. On the top strand, three of these four bases (A+1, G+3, and G+4) were protected. By contrast, none of the four purines on the bottom strand were protected, and indeed two of them showed increased sensitivity to methylation. We cannot exclude the possibility that in the presence of divalent ions, the endonuclease-DNA complex undergoes a conformational change that brings these nucleotides into proximity to the enzyme. The asymmetry of the protein-DNA contacts is in contrast to the symmetrical interactions seen with the well-studied restriction endonuclease *Eco*RI (25, 28). The mechanistic implications of the asymmetry I-*Ppo*I-DNA complex are uncertain.

The binding of DNA by a protein may increase the sensitivity of some bases to modification. This increase is usually thought to be caused by a distortion of the DNA helix. Significant perturbations of the I-*Ppo*I target DNA can be deduced from the pattern of protection against DMS. Most striking is the hypersensitive adenine at position +5. Also evident is the increased sensitivity of adenine −4 and guanine −3. Numerous DNA binding proteins have been shown to disrupt the double helix of their DNA substrate (18). For example, upon binding, *Eco*RI untwists several base pairs, introducing three symmetrically placed kinks into the helix (28). *Eco*RV compresses the major groove of its target sequence and introduces a 50° kink at the center of the recognition sequence (56). DNAs containing noncognate sequences are readily bound by *Eco*RV in the absence of Mg^{2+}, but no kink is evident. Crystallography data in combination with kinetic studies on *Eco*RV suggest that the protein-induced kink may be necessary for the formation of a high-affinity binding site for Mg^{2+} which is required for cleavage (49, 56). Protein-induced bending has previously been correlated with hypersensitivity in footprinting studies (13, 52). It remains to be directly determined whether I-*Ppo*I creates significant bends in its target DNA molecule.

Like many restriction enzymes, I-*Ppo*I associated as a dimer in sedimentation and gel filtration. The observation that I-*Ppo*I is dimeric makes it easier to conceptualize how this small protein binds a segment of DNA which encompasses several turns of the DNA helix. However, we cannot rule out the possibility that I-*Ppo*I actually binds the DNA as a higher-order structure, possibly a tetramer. Each subunit of a restriction enzyme presumably recognizes the elements of symmetry within its recognition sequence. From the results of DMS protection studies, however, we are not led to believe that the I-*Ppo*I subunits play similar roles. X-ray diffraction of I-*Ppo*I cocrystallized with its homing site would best provide additional information about the protein-DNA interactions.

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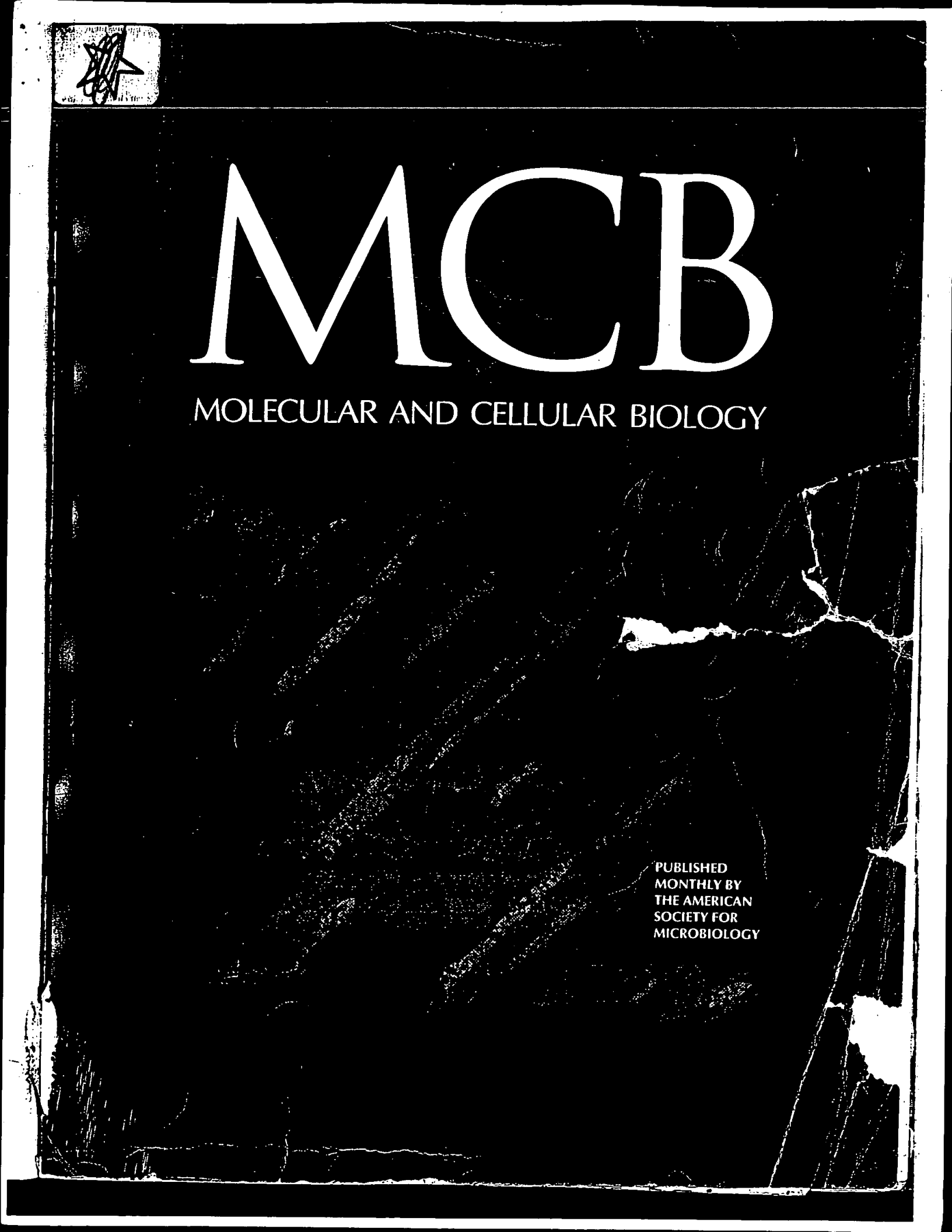
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